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Inactivation of *Six2* in mouse identifies a novel genetic mechanism controlling development and growth of the cranial base

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ABSTRACT

The cranial base is essential for integrated craniofacial development and growth. It develops as a cartilaginous template that is replaced by bone through the process of endochondral ossification. Here, we describe a novel and specific role for the homeoprotein Six2 in the growth and elongation of the cranial base. *Six2*-null newborn mice display premature fusion of the bones in the cranial base. Chondrocyte differentiation is abnormal in the *Six2*-null cranial base, with reduced proliferation and increased terminal differentiation. Gain-of-function experiments indicate that Six2 promotes cartilage development and growth in other body areas and appears therefore to control general regulators of chondrocyte differentiation. Our data indicate that the main factors restricting Six2 function to the cranial base are tissue-specific transcription of the gene and compensatory effects of other Six family members. The comparable expression during human embryogenesis and the high protein conservation from mouse to human implicate *SIX2* loss-of-function as a potential congenital cause of anterior cranial base defects in humans.

Introduction

The cranial base is part of the neurocranium, which surrounds and protects the brain. It develops as a cartilaginous template that is replaced by bone through the endochondral ossification process, when chondrocytes proliferate, secrete abundant extracellular matrix and terminate their differentiation process reaching the hypertrophic stage; thereafter, cells undergo apoptosis, blood invasion occurs and cartilage is eventually replaced by bone. Transition of the cells through these differentiation steps is strictly controlled both temporally and spatially (Wagner and Karsenty, 2001).

Ossification centers first emerge within the cartilage of the cranial base posteriorly and then anteriorly. The basioccipital ossification center is the first to appear starting at embryonic day 14 (E14) in mouse, followed by the basisphenoid and finally the presphenoid, formed between E16 and E17 (Shum et al., 2003). The cartilage segments separating these ossification centers are referred to as synchondroses. Analogous to the growth plate of long bones, synchondroses allow the rapid endochondral growth of the bone and eventually close when the bone reaches its final size. Moreover, chondrocytes in synchondroses and in the growth plate of long bones are distributed into resting, proliferating and hypertrophic zones. However, as a result of two hypertrophic and proliferating zones flanking a central resting zone, synchondroses produce a bidirectional growth with cartilage in the center and bone at each end, in contrast to the unidirectional growth from the growth plate of long bones.

The cranial base is essential for integrated craniofacial development and growth (Nie, 2005; Opperman et al., 2005). It has a dual embryonic origin, with its anterior portion deriving from cranial neural crest, while paraxial mesoderm forms the posterior one. These two embryonic tissues integrate to form the basisphenoid bone, which has a mixed embryonic origin. Defects that disrupt the growth and development of the cranial base result in phenotypes associated with several craniofacial syndromes, including achondroplasia, Apert syndrome, cleidocranial dysplasia, Crouzon syndrome and mandibulofacial dysostosis (Horowitz, 1981; Jensen and Kreiborg, 1993; Kjaer et al., 2001; Kreiborg et al., 1993; Peterson-Falzone and Figueroa, 1989; Tokumaru et al., 1996). In addition, malformations in the cranial base may be secondary to defects in the growth and development of other craniofacial skeletal and soft tissue structures, especially the cranial sutures, which develop in close association with the cranial base (Mooney et al., 1993; Perlyn et al., 2001).

Development and patterning of the cranial base depend on a number of transcription factors, among which Otx2, Gli2, Mfh1 and members of

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the Hox family (Barrow and Capecchi, 1999; Gendron-Maguire et al., 1993; Iida et al., 1997; Matsuo et al., 1995; Mo et al., 1997; Rijli et al., 1993). It is also controlled by members of key signaling pathways in craniofacial development, such as retinoic acid receptors (Lohnes et al., 1994; Mendelsohn et al., 1994) and platelet-derived growth factor (PDGF) receptor (Soriano, 1997). Endochondral bone formation in the cranial base is generally assumed to be controlled by the same molecular mechanisms that regulate development of the appendicular skeleton. Members of the Bmp family of proteins regulate chondrocyte proliferation and differentiation in long bones, and Bmp4 promotes chondrocyte proliferation and hypertrophy in the cranial base (Ducy and Karsenty, 2000; Hoffmann and Gross, 2001; Shum et al., 2003). Moreover, loss of function of chordin, an inhibitor of Bmp signaling, causes cranial base defects (Bachiller et al., 2003). Mutations in Fgfrs, fibroblast growth factor receptors, have been shown to lead to craniosynostosis and short limb skeletal dysplasia in humans (Rice et al., 2003), with some of these mutations primarily affecting cranial base growth (Chen et al., 1999; Chen et al., 2001; Eswarakumar et al., 2002). Similarly mice with loss of function of *Ihh*, a gene that regulates chondrocyte differentiation in the growth plate, display abnormal development of the synchondroses (Lanske et al., 1996; Vortkamp et al., 1996; Young et al., 2006). However, few studies have specifically explored the genetics of cranial base development and growth. The identification of cranial base-specific signaling pathways is essential in understanding the mechanisms of development, evolution and role in human disorders of this structure.

The *sine oculis*/*Six* family of genes encode for six transcription factors in vertebrates (Kawakami et al., 2000). Six proteins share a conserved homeodomain and a Six domain and can at least partially substitute for each other in vivo and in vitro (Ando et al., 2005; Giordani et al., 2007; Grifone et al., 2005). *Six* genes are diversely expressed during vertebrate embryogenesis and control the formation of various organs. In most cases, they positively regulate cells numbers within the developing embryo by promoting cell proliferation and/or survival and mainly act as transcription factors (Christensen et al., 2008; Kawakami et al., 2000). Six proteins act synergistically with Pax and Eya proteins in a regulatory network first identified in *Drosophila* as essential for compound eye formation. Six2 is required for kidney development, where it maintains the renal progenitor cell population in an undifferentiated state (Self et al., 2006). Six2 is also necessary for the formation of the pyloric sphincter (Self et al., 2009).

In this study, we describe a novel and specific role for Six2 in the development of the anterior cranial base. *Six2*-null newborn mice exhibit a shorter cranial base, where the bones are prematurely fused. Analysis of *Six2*-null mice shows that Six2 controls chondrocyte differentiation by promoting cell proliferation and preventing maturation into hypertrophic chondrocytes. Gain-of-function experiments show that Six2 similarly controls endochondral bone formation in other body areas, indicating that Six2 effects are likely to target general regulators of cartilage growth and differentiation. Tissue-specific transcription and compensatory effects from other *Six* genes restrict the *Six2*-null phenotype to the presphenoid component of the cranial base. The comparable expression during human embryogenesis makes *SIX2* a candidate for mutation in human disorders of this region of the cranial base.

Materials and methods

In situ hybridization and histological analysis

In situ hybridization was performed as described (Kanzler et al., 1998), using *Six2* (Oliver et al., 1995), *H4C* (Young et al., 2006), *ColX* and *ColI* probes (a kind gift from Christine Hartmann) and *osteopontin* (a gift from Ray Boot-Handford). TRAP staining was performed using a commercial acid phosphatase leukocyte kit (Sigma, St Louis, MO). Alcian blue staining of tissue sections was carried out as described (Pearse, 1968). For whole mount analyses, E18.5 embryos and newborns were eviscerated, skinned and fixed. Following fixation, cartilages were stained with Alcian blue and bones with alizarin red, as described (Mallo and Brandlin, 1997).

Mutant animals and microarray analyses

Animals experiments were carried out under ASPA 1986. *Six2* and *Six1; Six4*-null mutant mice have been described (Self et al. 2006; Grifone et al., 2005, respectively). Transient transgenic embryos expressing the *Hoxa2-Six2* transgene (abbreviated to *a2-Six2*) are described in Kutejova et al., 2005. Forelimbs of wild-type and transgenic E10.5 embryos analyzed by Affymetrix technology were derived from crossing a transgenic founder with no apparent phenotypic defects, which transmitted the *a2-Six2* transgene to the F2, causing perinatal lethality and the skeletal defects expected by overexpression of *Six2* (Kutejova et al., 2005). Dissected forelimbs were immediately frozen in liquid nitrogen. After genotyping the embryos, two pools were made of wild-type and transgenic forelimbs and total RNA was extracted from each using Trizol. Processing of the RNA and hybridization to Affymetrix 430A microarrays was performed according to the manufacturer's recommendations.

Human embryo collection and processing

Ethical approval for the studies was granted by the North West Regional Ethics Committee. Human embryos were collected with informed consent under guidelines issued by the UK Government Polkinghorne Committee, fixed, embedded and sectioned as described previously (Hanley et al., 2008; Piper et al., 2004).

Results

Craniofacial abnormalities in Six2-null mice

Six2-null mice die at birth (Self et al., 2006) and are easily distinguishable from their heterozygous and wild-type littermates by their abnormal head shape.

Skulls from wild-type and *Six2*-null newborns were stained with Alcian blue and Alizarin red to visualize cartilage and bone, respectively. The mutant skulls appeared compressed as compared with the wild type (Figs. 1A, B). A ventral view of the wild-type cranial base revealed basioccipital, basisphenoid and presphenoid bones well separated by cartilage segments, whereas no cartilage segments between the basioccipital and basisphenoid bones and between the basisphenoid and presphenoid bones were seen in *Six2*-null newborns (Figs. 1C, D). The abnormal growth of the cranial base in the mutant did not affect formation of the palate, located below the anterior portion of the cranial base (not shown). The shorter appearance of *Six2*-null skulls is entirely caused by the premature fusion of the bones in the cranial base, as the mutant mandible developed correctly (Figs. 1E, F).

The nasal bones were present but appeared delayed in development in *Six2*-null compared to wild-type (not shown). The rest of the skull (skull vault, occipital and parietal regions) developed normally.

Third and fourth branchial arch-derived cartilages were hypomorphic in *Six2*-null newborns. The thyroid cartilage was smaller and the inferior horns failed to extend as they did in the wild type (Figs. 1G, H, arrow). The cricoid cartilage was also smaller in *Six2*-null newborns and its dorsal components did not form (not shown).

Six2-null newborns did not display any of the craniofacial defects of $Six1^{-/-}$ and $Six1;Six4^{-/-}$ (e.g. shorter mandible, absence of the retro-tympanic process of the temporal bones, poorly developed otic capsule with malformations in the associated middle ear ossicles) (Grifone et al., 2005; Laclef et al., 2003). However, mild defects in first and second branchial arch derivatives of *Six2*-nulls were observed with incomplete penetrance and included malformation of the styloid

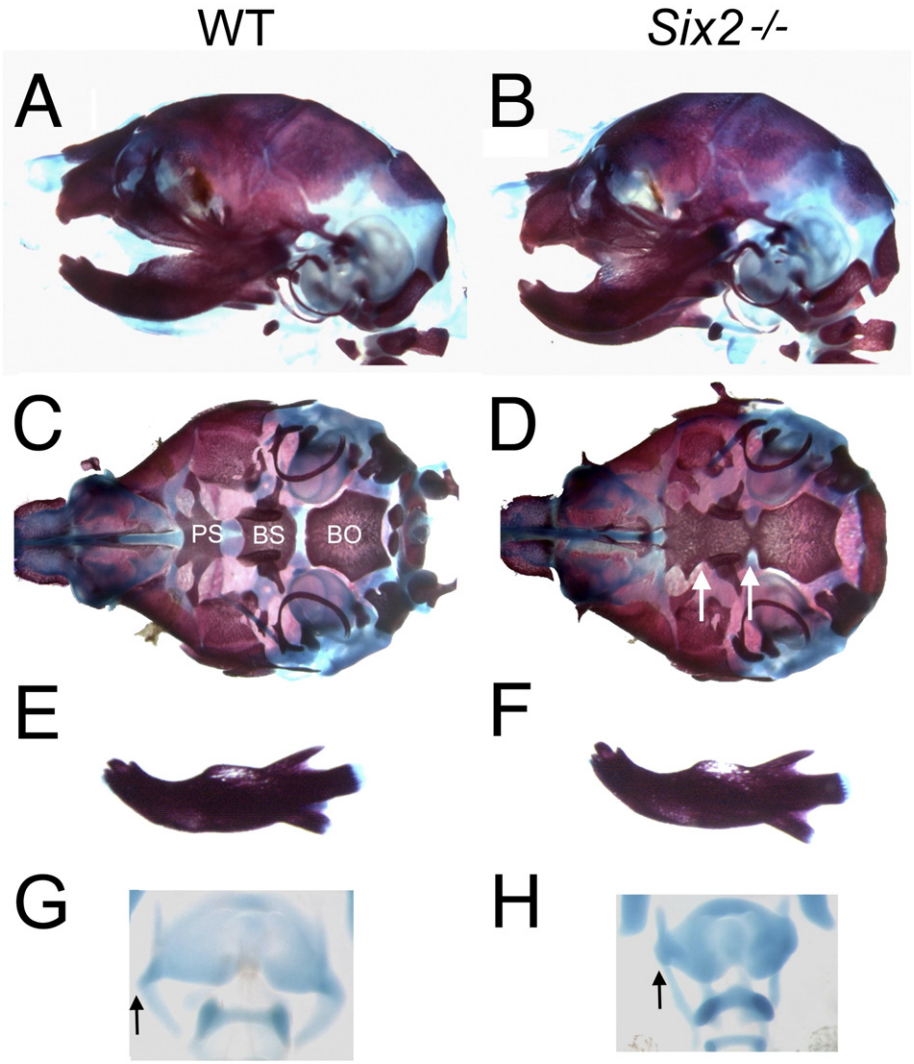

Fig. 1. Craniofacial phenotype of *Six2*-null newborns. Skeletal preparation of wild-type (A, C, E, G) and *Six2*-null (B, D, F, H) newborns. Lateral view of the wild-type head (A) and the *Six2*-null shorter head (B). Ventral view of the wild-type (C) and *Six2*-null (D) head shows absent intrasphenoidal and reduced spheno-occipital synchondrosis (white arrows between presphenoid and basisphenoid bones and basisphenoid and basioccipital bones, respectively) in the mutant. Palatal bones and mandible were removed to expose the cranial base. Mandibles isolated from wild-type (E) and *Six2*-null (F) newborns. Thyroid cartilages isolated from wild-type (G) and *Six2*-null (H) newborns; arrow indicates the inferior horn of the thyroid cartilage. PS, presphenoid; BS, basisphenoid; BO basioccipital bones.

process and of the manubrium and the processus brevis of the malleus (1/5 and 2/5 *Six2*-null newborns respectively) (data not shown).

Six2-nulls display premature chondrocyte maturation

The cranial base is built as a cartilage template, which is replaced by bone through the process of endochondral bone formation. In this multi-step process, chondrocytes proliferate, exit the cell cycle, undergo hypertrophy and ultimately die while invading osteoblasts deposit a new, bone-specific matrix. The premature ossification observed in the *Six2*-null cranial base could be caused by disturbance in any of the above steps; we analyzed earlier developmental stages to determine the primary cause of the shorter cranial base of *Six2*-nulls.

Alcian blue staining of longitudinal sections of E16.5 wild-type and mutant heads identified the developing presphenoid (PS) bone as the element most affected (Figs. 2A, B), with a shorter cartilage precursor and an expanded area of large hypertrophic cells in the mutant (Figs. 2C, D), suggesting an accelerated cell differentiation process. In keeping with these findings, the region positive for type X collagen (*ColX*) expression, a marker of hypertrophic chondrocytes, was wider in the PS mutant precursor (Figs. 2E, F). No obvious differences between wild-type and mutant mice were observed in type I collagen (*ColI*) in situ hybridization in the presphenoid bone precursor (Figs. 2G, H), suggesting that the absence of Six2 does not affect early differentiation of osteoblasts. Finally, Alizarin red staining revealed comparable ossification in the E16.5 wild-type and mutant cranial base; the basioccipital ossification center was visible, but no ossification could be detected in the PS bone precursor, suggesting the process of ossification does not begin prematurely in *Six2*-nulls (Figs. 2I, L). Once chondrocytes become hypertrophic, they are fated for rapid cell death and replacement by bone (de Crombrugghe et al., 2001). One day later (E17.5), ossification was detected in the wild-type and mutant anterior cranial base, as revealed by the presence of *ColI*-positive osteoblast cells (Figs. 3C,D). As a result of the expanded domain of hypertrophic cartilage observed earlier, a larger area of the *Six2*-null anterior cranial base underwent ossification compared to wild-type. *Six2*-null osteoblasts correctly expressed *osteopontin* and revealed alkaline phosphatase activity (Figs. 3F,H). Osteoclasts, identified by tartrate resistant acid phosphatase (TRAP) activity, were present in the area undergoing ossification in wild-type and mutant anterior cranial base (Figs. 3I,L).

These observations indicate that the extensive ossification observed in the *Six2*-null cranial base is caused by an abnormal chondrocyte differentiation, leading to expanded areas of hypertrophic cartilage (ready for bone replacement). Despite the increased size of the areas

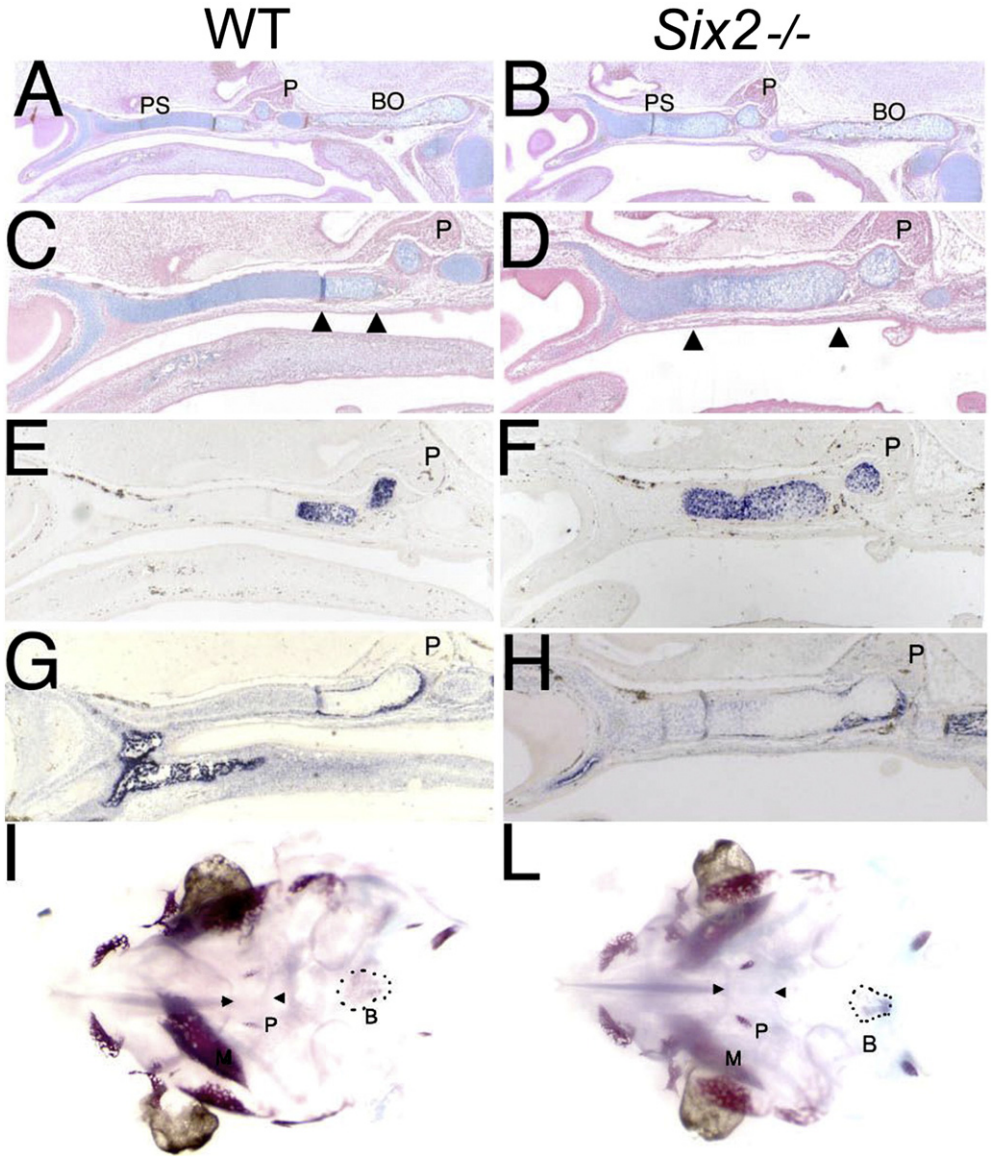

Fig. 2. Premature chondrocyte differentiation in *Six2*-nulls. Adjacent parasagittal sections from E16.5 wild-type (A, C, E, G) and *Six2*-null (B, D, F, H) mice were stained with Alcian blue (A–D) and analyzed for *ColX* (E,F) and *ColI* (G,H) expression by in situ hybridization. (A, B) Sections show the entire cranial base. (C–H) Higher magnification of wild-type and mutant presphenoid bone precursor. (C, D) A larger area of hypertrophic cells (arrowheads) is present in *Six2*-null presphenoid precursor. (E, F) *ColX* expression identifies large cells as hypertrophic chondrocytes. (G, H) Blue staining (*ColI*) around the edge of the PS bone precursor corresponds to the area undergoing ossification. PS, presphenoid; BO, basioccipital bones; P, pituitary gland. (I, L) Alizarin red-stained skeletal preparation of E16.5 wild-type (I) and *Six2*-null (L) embryos. The basioccipital ossification center is enclosed in a dotted circle. Arrowheads mark the expected position of the PS ossification center. M, mandibular bone; P, palatal bone.

undergoing ossification, the ossification process itself appears to be normal in the absence of Six2.

The absence of Six2 reduces chondrocyte proliferation

The increased number of mature cells observed in the PS region of *Six2*-nulls at E16.5 may reflect an overall acceleration of the process of endochondral bone formation. To verify this possibility, we followed endochondral bone formation from its early stages.

Alcian blue staining of E13.5 wild-type and *Six2*-null cranial base outlined early, weakly stained PS chondrogenic condensations, as the process of matrix deposition is still in progress at this stage (compare Figs. 4A, B with the more robust staining observed 1 day later in Figs. 4E, F). The size of the PS condensations and the deposition of extracellular matrix appeared comparable in wild-type and *Six2*-null embryos (Figs. 4A, B). Similarly, analysis of proliferating cells, based on expression of *H4C*, a universal marker of proliferating cells (Young et al., 2006), displayed no significant difference between the wild and the mutant PS chondrogenic condensations (mean percentage of proliferating cells was 13% and 11% in the wild-type and mutant PS precursor respectively; P not significant) (Figs. 4C, D), suggesting early stages of endochondral bone formation are not affected in *Six2*-null embryos.

One day later (E14.5), analysis of proliferating cells revealed the earliest observable difference between the wild-type and the *Six2*-null cranial base. A significant reduction in the number of proliferating cells was detected in *Six2*-null PS precursor compared to wild type (Figs. 4G, H, Q). The E14.5 wild-type and *Six2*-null PS precursor displayed a similar morphology (Fig. 4E, F) and, as expected for this developmental stage (Shum et al., 2003), no differentiated *ColX*- positive cells were detected in the E14.5 wild-type or mutant PS precursor. These cells were only detected in the basioccipital precursor in the posterior cranial base (not shown).

Alcian blue staining of E15.5 presumptive PS bones revealed few morphological differences between wild-type and *Six2*-null embryos at this stage. The mutant PS bone appeared wider, displayed a slight increase in large hypertrophic chondrocytes (Figs. 4K, L), and the

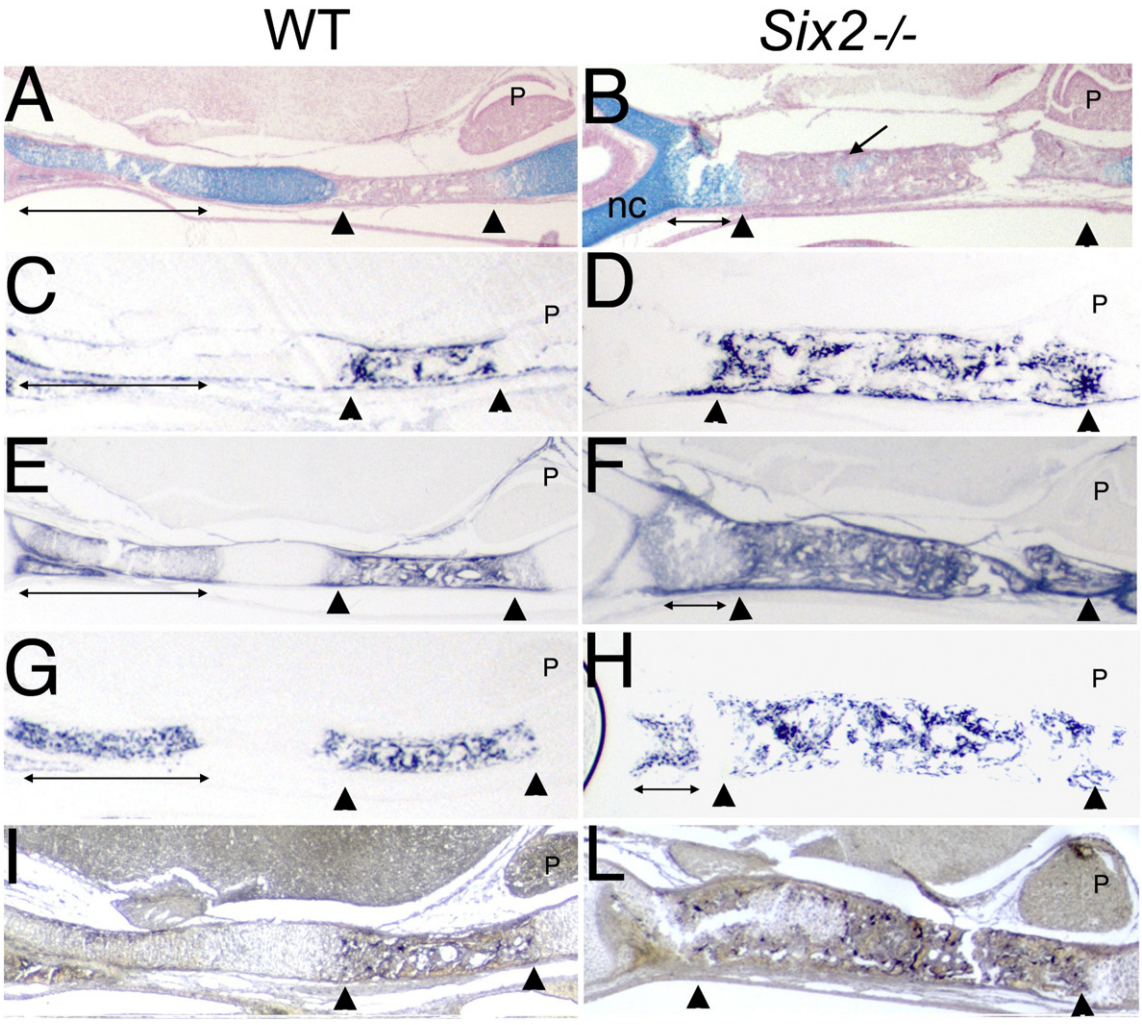

Fig. 3. Ossification in *Six2*-nulls. Adjacent parasagittal sections from E17.5 wild-type (A, C, E, G, I) and *Six2*-null (B, D, F, H, L) anterior cranial base were stained with Alcian blue (A,B) and analyzed for *Coll* expression (C,D), alkaline phosphatase activity (E,F), *osteopontin* expression (G,H) and TRAP activity (I,L). (A, B) Cartilage is almost completely absent in the mutant PS, with the exception of small isolated areas (black arrow). (C, D) A wider domain of ossification, marked by *Coll*-positive cells, is present in *Six2*-null PS precursor (arrowheads). (E–H) Alkaline phosphatase activity and *osteopontin* expression are detected in osteoblasts (arrowheads) and terminally differentiated chondrocytes (left right arrow). (I, L) TRAP-positive osteoclasts are detected in the areas undergoing ossification in both the wild-type and mutant anterior cranial base (dark brown precipitates in I, L). Nc, nasal cartilages; P, pituitary gland.

ColX-expression domain occupied a slightly larger area in the mutant as compared to the wild-type (Figs. 4M, N). Analysis of the number of proliferating cells at E15.5 identified *H4C*-expressing cells only in the anterior third of the PS bone precursor in the mutant embryo but in the anterior two-thirds of the corresponding wild-type structure (Figs. 4O, P, Q). In addition, in the wild-type PS precursor, the area occupied by proliferating cells extended to the area harboring *ColX*-expressing mature cells, whereas the mutant structure showed an Alcian blue-stained area that was neither *H4C*- nor *ColX*-positive, in which cells stopped proliferation but did not proceed to differentiate (Figs. 4O, P). The expanded area of hypertrophic chondrocytes observed in the E16.5 PS bone precursor (Figs. 2E, F) suggests that these cells enter premature differentiation. The distribution of TUNEL-positive cells was similar in the wild-type and mutant *Six2*-null PS precursor at this or at an earlier stage (E14.5), indicating that increased cell death does not contribute to the shorter cranial base of *Six2*-null (data not shown).

These data show that at early stages (E14.5), when no morphological differences between the wild-type and mutant bone precursors are detected, the pool of proliferating chondrocytes is considerably reduced in the *Six2*-null PS precursor. Given that chondrocyte proliferation and maturation are tightly coupled processes (Sorrentino et al., 1990; Tao and Umek, 2000; Umek et al., 1991), the increased number of mature cells observed in the PS region of *Six2*-nulls at E16.5 may arise secondary to a deranged proliferation. Thus, defective chondrocyte proliferation appears to be the primary cause of the shorter cranial base in *Six2*-null mice.

How is Six2 function restricted to the PS precursor?

Tissue-specific transcription represents an important mechanism in spatially restricting the function of a protein. In an effort to clarify how Six2 function is restricted to the anterior cranial base, we investigated the distribution of *Six2* expression in the developing head region. At E13.5, *Six2* expression was detected in the nasal epithelia and cartilages, pituitary gland and in the PS and basisphenoid bone precursors as well as in the Meckel cartilage and the thymus (Fig. 5A). At E14.5 *Six2*-expressing cells were still present in the mid-posterior region of the PS (Fig. 5B, arrowheads) and labeled mostly the outer sides in the anterior region (Fig. 5B). The presence of proliferating, *H4C*-expressing cells in the mid-posterior region of the PS bone precursor (Fig. 5C, arrowheads; see also Fig. 5G) supports the possibility of a direct effect of Six2 on cell proliferation.

The data above suggest that *SIX2 is* an excellent candidate for mutation as the underlying cause of human cranial base congenital defects. To further investigate this possibility, we examined *SIX2* expression in the

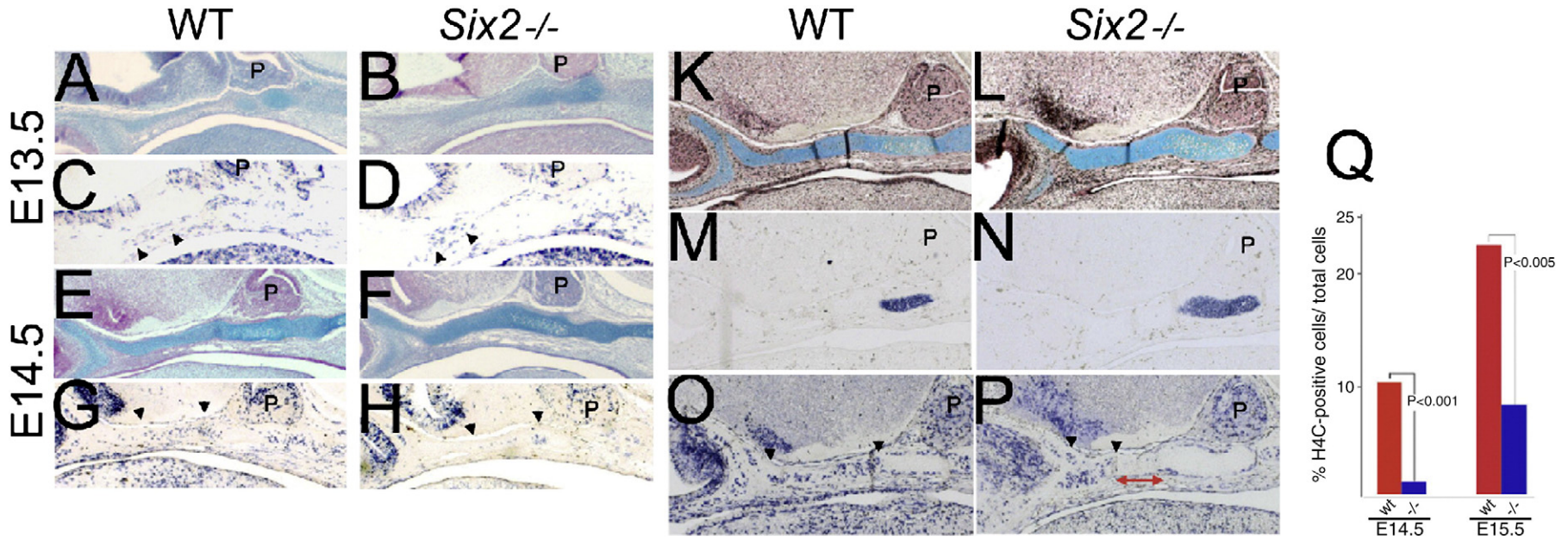

Fig. 4. Decreased chondrocyte proliferation in *Six2*-null PS precursor. Adjacent parasagittal sections of wild-type (A, C, E, G, K, M, O) and *Six2*-nulls (B, D, F, H, L, N, P) anterior cranial bases were stained with Alcian blue (A,B, E,F, K, L) and analyzed for *H4C* (C, D, G, H, O, P) and *ColX* (M,N) expression by in situ hybridization. At E13.5 (A–D) wild-type and mutant appear similar by Alcian blue staining (A, B) and *H4C* expression (C, D, arrowheads). E14.5 (E–H). Alcian blue staining reveals a comparable morphology in wild-type (E) and mutant (F); *H4C* expression by in situ hybridization reveals a reduction in proliferating cells in the mutant (G, arrowheads) compared to the wild-type (H, arrowheads). E15.5 (K–P). Alcian blue staining and *ColX* expression demarcate a slightly larger area of hypertrophy in the mutant (L, N) than in the wild-type (K, M). Proliferating cells, marked by *H4C* expression, occupy a considerably larger portion of the PS precursor in wild-type (O, arrowheads) than in the mutant (P, arrowheads). The mutant PS exhibits an area (red arrows) negative for both *H4C* and *ColX*. P, pituitary gland. Q: Quantification of proliferating cells within the PS precursor of E14.5 and E 15.5 wild-type (red) and mutant (blue) embryos. Proliferating cells are expressed as the ratio of *H4C*-expressing cells within the total number of cells counted in the PS precursor. Data represent the mean percentage of 7 independent sections of the PS precursor, derived from at least 2 different embryos for each genotype and each embryonic stage.

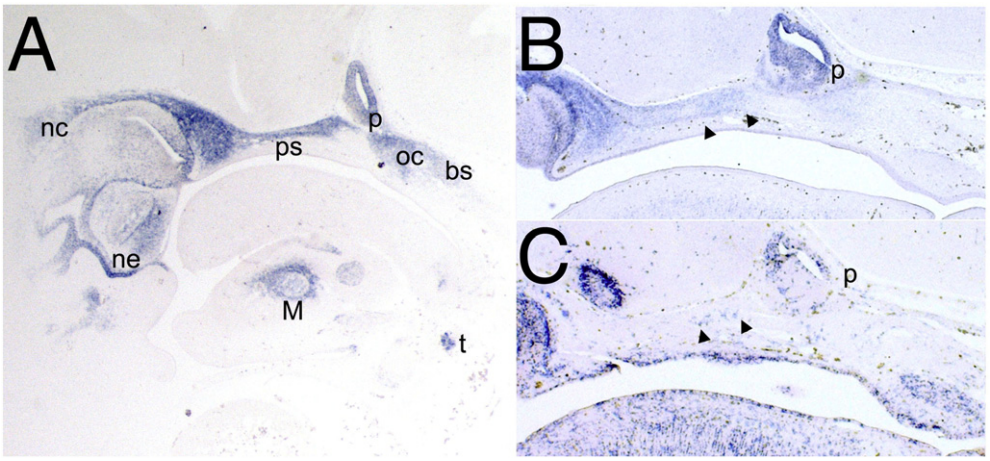

Fig. 5. *Six2* expression in the head region. Parasagittal sections from E13.5 (A) and E14.5 (B, C) wild-type embryo analyzed for *Six2* (A, B) and *H4C* (C) gene expression by in situ hybridization. (A) *Six2*-positive cells are present in the nasal cartilages and epithelia, the pituitary gland, the PS precursor, the otic capsule, the basisphenoid bone precursor, the Meckel cartilage and the thymus. (B) Higher magnification of *Six2* expression in the E14.5 PS precursor. *Six2*-expressing cells are detected in the mid-posterior region of the PS (Fig. 4B, arrowheads) and mostly at the outer sides in the anterior region of the PS. (C) Adjacent section to B hybridized with *H4C* probe to identify *H4C*-positive, proliferating cells (marked by arrowheads). bs, basisphenoid; M, Meckel cartilage; ne, nasal epithelia; nc, nasal cartilages; oc, otic capsule; p, pituitary gland; ps, presphenoid; t, thymus.

developing human embryo at 48 days post-conception (dpc; Carnegie stage 19) chosen to correspond to E13–13.5 of mouse development. *SIX2* transcripts were apparent in the anterior cranial base with detection concentrated in the sphenoid precursor (Fig. 6B, arrowheads). *SIX2* expression was also detected in the pituitary gland (Fig. 6B).

Six2 has a general effect on endochondral bone formation

In a previous study to examine the role of *Six2* in the generation of the *Hoxa2* mutant phenotype (Kutejova et al., 2005), we showed that *Six2* overexpression directed by the *Hoxa2* enhancer (Nonchev et al., 1996) resulted in the formation of ectopic cartilage, with abnormal elongation of the lesser horn of the hyoid bone and the styloid process, e.g. the cartilages patterned by *Hoxa2*. Here we show that the enhancer also directs *Six2* expression in the forelimbs and the somitic mesoderm (Figs. 7A, B) in addition to the expected ectopic expression in the second branchial arch. Skeletal preparation of E18.5 transgenic embryos overexpressing *Six2* revealed an abnormal phenotype in the forelimbs (6/10 transgenic embryos). Consistent with the detection of ectopic *Six2* in the anterior limb bud (Fig. 7B, arrow), the radius was the most affected element, being shorter and displaying failure of ossification (Figs. 7C, D; $n=6$). In the embryos with the most extreme phenotype, the humerus was also shorter (Fig. 7D; $n=3$) and formation of ectopic cartilage was detected (Fig. 7D, arrow; $n=2$). Delayed ossification was also observed in the vertebral bodies (Figs. 7E, F; $n=3$), consistent with *Six2* overexpression in the somites.

These effects are opposite to those observed in the cranial base of *Six2*-null mice. *Six2* gain-of-function halts the replacement of cartilage by bone, while its absence accelerates ossification. High levels of *Six2* induce the formation of ectopic cartilage in the forelimbs and expand branchial arch cartilages, defects consistent with enhanced chondrocyte proliferation, while the absence of Six2 impairs proliferation of chondrocytes in the PS precursor. These opposing phenotypes indicate that Six2 function is not context specific but can generally affect growth and development of cartilage in the endochondral skeleton.

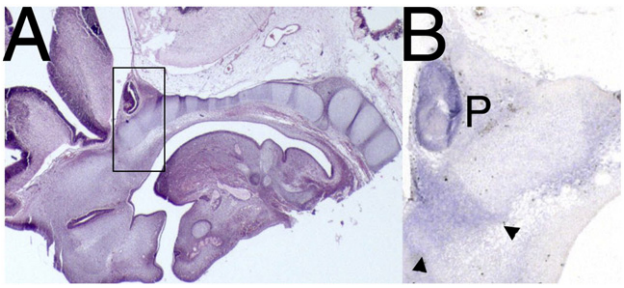

Fig. 6. *SIX2* is expressed in the sphenoid precursor. Parasagittal adjacent sections from 48 dpc human embryo HE stained (A) and analyzed for *SIX2* gene expression by in situ hybridization (B). (B) Higher magnification of the boxed area in A shows *SIX2*-positive cells in the sphenoid bone precursor (arrowheads). P, pituitary gland.

Cranial base defects in Six1;Six4 double mutants

The increasing severity of compound mutants of *Six* genes indicate that Six family members act redundantly. The craniofacial defects observed upon inactivation of *Six1*, the closest homologue of *Six2*, are enhanced in *Six1;Six4* double mutants (Grifone et al., 2005). We investigated whether skeletal preparations of E18.5 *Six1;Six4*-null mice show a similar cranial base phenotype to *Six2* mutant, as this would suggest possible redundancy. Analysis of *Six1;Six4*-null heads displayed an abnormal cranial base with premature fusion of the exoccipital and basioccipital bones (Fig. 8B). Both basioccipital and basisphenoid bones were malformed and displayed a partial unilateral fusion. However, the presence of a large cartilage segment to separate basisphenoid and presphenoid bones (Fig. 8B, arrow) indicates that ossification of this area occurs normally in the absence of Six1 and Six4 proteins. These findings indicate that Six2 controls development of the anterior cranial base with no or little redundancy from other family members and may help to explain the restricted phenotype, mainly impacting the PS precursor, observed upon Six2 inactivation.

Discussion

The identification of cranial base-specific pathways is essential to clarify the mechanisms of cranial base development and the role of the cranial base in human disorders. Here we uncover a new role for the homeoprotein Six2 in the growth and development of the cranial base.

Six2-null newborns present a shorter cranial base due to a premature fusion of the cranial bones. The defect is more accentuated in the anterior part of the cranial base, with the phenotype originating in the presphenoid bone precursor. Histological analysis shows a normal cranial base in E13.5 *Six2*-null mutants, indicating that the absence of Six2 does not affect early steps of cranial base development, such as migration of cranial neural crest, and condensation and differentiation of mesenchymal progenitors. Cell proliferation appears normal in the E13.5 mutant PS chondrogenic condensation; 1 day

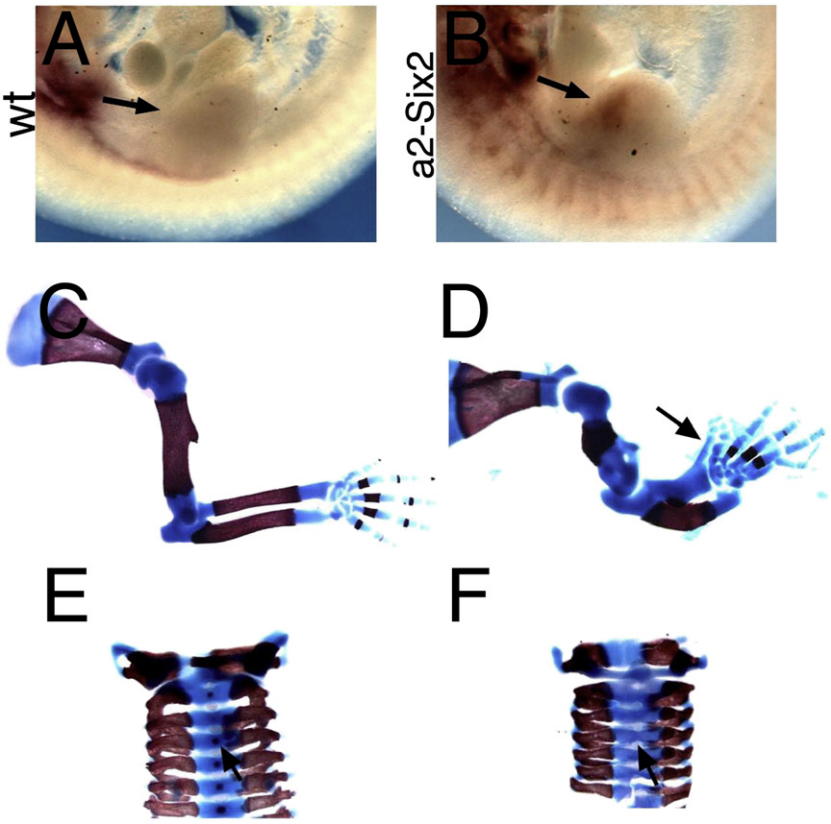

Fig. 7. Broad effect of *Six2* on endochondral bone formation. (A) Expression of *Six2* in E10.5 wt embryos. *Six2* mRNA is not detected in the limb bud (arrow). (B) *Six2* is ectopically expressed in the anterior limb bud (arrow) and the somitic mesoderm of transgenic embryos. (C) Forelimb skeleton of E18.5 wt embryo. (D) Forelimb skeleton of a transgenic littermate. The radius exhibits a complete lack of ossification and an ectopic cartilage extends to the side of the hand (arrow). The ulna and the humerus are malformed. (E) Cervical vertebrae of E18.5 wt embryo. The red dots in the wild-type vertebral bodies are areas of ossification (arrow). (F) Cervical vertebrae of a *Six2*-overexpressing transgenic littermate, in which ossification is absent (arrow).

later, however, the pool of proliferating chondrocytes is drastically reduced in the *Six2*-null PS precursor. Reduced proliferation is followed at a later stage (E16.5) by an increased number of chondrocytes entering terminal differentiation. After reaching terminal differentiation, chondrocytes are physiologically fated for rapid cell death and hypertrophic cartilage is replaced by bone (de Crombrugghe et al., 2001). The abnormal chondrocyte differentiation observed in the *Six2*-null cranial base may lead to more extensive ossification by expanding the areas of hypertrophic cartilage. As cartilages are sites of active growth during endochondral ossification, their premature disappearance interferes with the correct elongation of the cranial base and causes the fusion of the bones. Therefore, accelerated chondrocyte differentiation may directly explain the defects observed in the *Six2*-null cranial base.

Six2 may independently act to regulate proliferation first and then terminal differentiation of the cells into hypertrophic chondrocytes. However, as chondrocyte proliferation and maturation are tightly coupled processes (Sorrentino et al., 1990; Tao and Umek, 2000; Umek et al., 1991), a primary defect in proliferation is likely to impact on terminal differentiation. Thus, defective proliferation of chondrocytes within the presphenoid bone precursor appears to underlie the shorter cranial base in *Six2*-null mice.

The failure of *Six2*-nulls to form kidneys reflects a depletion of undifferentiated renal progenitors due to increased death and premature differentiation of these cells (Self et al., 2006). While cells in the cranial base also differentiate prematurely, the earliest defect observed in the cranial base is a change in the number of proliferating cells, possibly leading to the premature differentiation. Furthermore, increased cell death is not detected in the mutant cranial base. These observations and the absence of proliferation defects in *Six2*-null kidneys, suggest a different role for Six2 in the presphenoid bone precursor than in the kidney.

How does Six2 control growth and development of the PS bone? One can envision two possible alternative scenarios. In the first case, Six2 uniformly controls cell proliferation in the developing PS precursor. Its absence generally decreases cell proliferation and accelerates terminal differentiation of chondrocytes (this possibly as a secondary effect). Proliferation and differentiation are abnormally uncoupled in the E15.5 mutant PS precursor, where cells are held in between the proliferative and the differentiation step (Fig. 4P, red double arrow). Alternatively, Six2 may control cell proliferation, but only in a restricted area of the PS bone precursor. At all the stages examined, starting from E14.5, defects are consistently observed in the mid-posterior region of the PS precursor. At E14.5, proliferating cells preferentially localize in the mid-posterior region of the wild-type PS precursor, while very few cells are dividing in the corresponding area of the mutant. At E15.5, the same area displays no proliferating cells in *Six2*-nulls, while a similar density of proliferating cells is observed in the anterior area of the E15.5 wild-type and mutant PS precursor (arguing against a general defect in proliferation) (Fig. 4P, arrowheads). At E16.5, cells differentiate prematurely in a corresponding mid-posterior position of the mutant PS precursor. These observations suggest that cells in the mid-posterior region of the PS precursor do not proliferate and enter terminal differentiation prematurely in the absence of Six2. The hypothesis of a spatially restricted defect within the PS precursor explains the presence of cells held in between proliferation and terminal differentiation in terms of a defective cell population within a unit (PS precursor), which otherwise develops "normally". In both cases (i.e., if Six2 were to control cell proliferation in the entire PS precursor or in a limited cell population of the PS precursor), Six2 absence would eventually impact on the

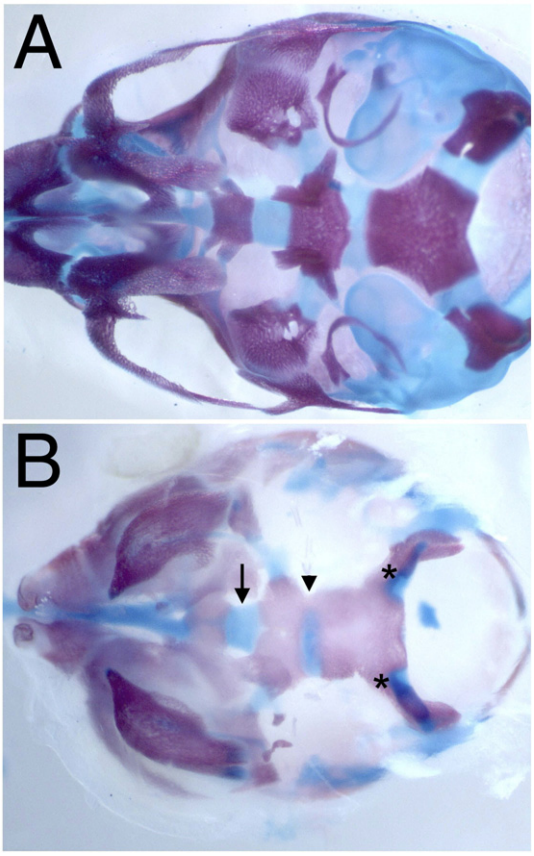

Fig. 8. Cranial base phenotype of E18.5 *Six1;Six4* double mutant. Skeletal preparation of wild-type (A) and *Six1;Six4* -null mutant (B). Ventral view of the head shows fusion between the exoccipital and basioccipital bones (asterisks in B) and malformed basioccipital and basisphenoid with partial unilateral fusion (arrowhead). Unlike *Six2*-null, *Six1;Six4* double mutant show a large intrasphenoidal synchondrosis (arrow). In panel A, mandibles were removed to expose the cranial base.

general growth of the PS bone, as the PS cartilage is spatially and temporally organized into a functional unit.

Six2 is widely expressed in the E13.5 PS precursor, but at E14.5 Six2 expression is mainly detected in the mid-posterior region. Proliferating cells are present in this area of the PS precursor at E14.5 and, as a transcription factor, Six2 could directly control the proliferation of these cells.

Some additional points remain open for discussion. Firstly, there is a temporal delay between reduced cell proliferation, first observed at E14.5, and the change in size and morphology of the mutant PS precursors, detected at E16.5. This can be explained by the observation that the percentage of proliferating cells in the PS precursor increases over development (with about 10% of the PS precursor cell population proliferating at E14.5, followed by almost 25% at E15.5). Therefore, although striking differences are observed in numbers of proliferating cells between the E14.5 wild-type and mutant PS precursors, the population of proliferating cells (affected in the mutant) may not be large enough to have an immediate effect on the morphology of the mutant PS precursor. Secondly, although Six2 is highly expressed in the E13.5 precursor and proliferating cells are present at this stage, the absence of Six2 appears not to affect cell proliferation until E14.5. One can speculate that the presence of other genes, which control development and growth of the PS precursor together with Six2, may partially (before a certain stage) compensate Six2 absence. The aforementioned possibility that Six2 may selectively control chondrocyte proliferation and differentiation in restricted areas of the PS precursor, offers a possible explanation to the observed late onset of the phenotype.

To address the question of how Six2 controls chondrocyte proliferation, we tested whether Six2 exerts a general effect on endochondral bone formation or, instead, functions upstream of genes selectively acting in the head. We found that overexpression of *Six2* impacts the formation of cartilage in all the embryonic areas where ectopic expression was observed and generates opposite effects to those observed in the cranial base of *Six2*-null mice. This broad effect of Six2 on cartilage development and differentiation, together with the high penetrance of the phenotype in transgenic embryos, strongly argues against the possibility that Six2 effects are restricted to the cranial base by regulation of genes specifically involved in the development of this region. Instead, the findings suggest that Six2 directly controls general regulators of chondrocyte differentiation. In these transgenic embryos, *Six2* ectopic expression does not persist throughout endochondral bone formation. High and spatially restricted levels of *Six2* are observed at E10.5 and decline at E11.5. Considering that active chondrogenesis is in progress throughout the embryo at E11.5 (Zhou et al., 1998), it is likely that Six2 affects early stages of endochondral bone formation and that the defective ossification results from disturbing the onset of a process relying on a series of tightly linked steps.

The phenotype of *Six2*-overexpressing transgenic embryos is similar to that observed when *Sox9*, a master regulator of cartilage and the gene responsible for maintaining proliferating chondrocytes, is overexpressed in chondrocytes (Akiyama et al., 2004). Overexpression of *Sox9* delayed endochondral ossification since bone formation from a cartilage template requires that chondrocytes switch off *Sox9* to become hypertrophic and eventually die, thus allowing osteoblasts to invade the cartilage and deposit bone matrix. Moreover, *Sox9* overexpression generated fusion of the hyoid to the styloid process, a defect similar to the one observed in *Six2*-overexpressing transgenic embryos (Kutejova et al., 2005). While the detection of *Sox9* upregulation in *Six2*-overexpressing embryos (NB, unpublished results) raises the possibility that Six2 acts directly upstream of Sox9, increased *Sox9* expression might simply reflect the increase in the pool of proliferating chondrocytes upon *Six2* overexpression. Indeed measurement of *Sox9* mRNA levels in different cell lines upon overexpression of *Six2* revealed no change (KPH and NB, unpublished results), suggesting *Sox9* is not a direct mediator of Six2 function.

Based on previous findings linking Six2 and Six5 to the control of *Igf2* and *Igfbp5* (Kutejova et al., 2008; Sato et al., 2002), we examined the possibility that Six2 may control the IGF axis. We found that *Igf2* expression is downregulated in the mutant PS precursor at E14.5, when the cellular phenotype is first observed. *Igf2* was also detected among the genes upregulated upon *Six2* overexpression by expression profiling of E10.5 wild-type and transgenic forelimb buds (NB, unpublished results). Altogether these observations point at *Igf2* as a likely functional target of Six2 and further investigation will be directed to establish its functional role downstream of Six2.

Finally, it has been recently reported that chondrocyte-specific, conditional inactivation of Dicer, an essential component for the biogenesis of small noncoding microRNAs (miRNAs), simultaneously decreases chondrocyte proliferation and accelerates differentiation of the cells into postmitotic hypertrophic chondrocytes (Kobayashi et al., 2008). The cellular phenotype described in Dicer-null growth plates bears similarity with the one observed in the *Six2*-null cranial base, suggesting a possible link between the molecular function of Six2 and the regulatory mechanisms operated by miRNAs.

In contrast to the broad effect of *Six2* overexpression on cartilage development and differentiation, the absence of Six2 causes extremely localized skeletal defects and affects only the growth and development of the anterior cranial base and of the thyroid and cricoid cartilages. The main factor responsible for spatially restricting Six2 function is its limited transcription in the developing embryo. This mechanism cannot entirely account for Six2 localized function as *Six2* is expressed in other areas destined to form cartilages (Meckel, nasal and otic capsule, basisphenoid precursor), which develop normally in its absence.

Redundancy across Six family members is another mechanism that most likely contributes to limiting Six2 function to specific areas of the developing embryo. A redundant function of Six2 in the craniofacial area is supported by the observation that *Six2*-null embryos display incompletely penetrant defects in first and second arch skeletal structures, which are strongly affected in *Six1* and *Six1;Six4* mutants (Grifone et al., 2005; Laclef et al., 2003). We have reported here that the anterior cranial base develops normally in *Six1;Six4* mutants, indicating that Six2 controls development of the anterior cranial base with no or little redundancy from other family members. This observation provides an explanation for the severe phenotype observed in the anterior cranial base upon *Six2* inactivation. It should also be considered that the anterior cranial base is cranial neural crest-derived, as opposed to the mesoderm-derived posterior cranial base. This heterogeneity may result in a different sensitivity to the absence of Six2 of the anterior cranial base versus the posterior cranial base, and eventually to the development of a more severe phenotype.

The brachyrrhine (*Br*) mouse mutant, generated in a neutron irradiation experiment (Searle, 1966), displays an abnormal growth pattern of the skull in the anterior cranial base (Lozanoff et al., 1994). Linkage analysis has mapped the *Br* mutation in the proximity of the *Six2* locus (Fogelgren et al., 2008). Although sequencing of the *Six2* gene, including the promoter region, did not identify any mutation, *Six2* expression was found to be severely reduced in the *Br* mutant. In contrast to the *Six2*-null mutant, whose presphenoid bone precursor exhibits premature ossification, the *Br* mutant phenotype is characterized by complete absence of the presphenoid bone. These different phenotypes suggest that the *Br* mutation could affect expression of other genes in addition to *Six2*. *Six3* is a potential candidate, since the gene lies close to *Six2*.

Craniofacial abnormalities, including brachicephaly, have been observed in patients with a deletion encompassing *SIX2* (Rajcan-Separovic et al., 2007). Our findings, and the presence of *SIX2* in the anterior cranial base in humans, may link *SIX2* to human congenital disorders caused by an abnormal growth of the cranial base.

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